

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph at page 2, lines 6-14, with the following amended paragraph:

Semaphorin 4B (hereinafter sometimes briefly referred to as SEMA4B) having 41% homology with Semaphorin 4D on an amino acid level is registered in ~~GenBank~~-GENBANK™ as a putative gene from the genome sequence (~~GenBank~~-GENBANK™ Accession No. XM_044533). SEMA4B is reported as one of genes overexpressed under hypoxic conditions (WO 02/46465). It is further reported that several hundreds of base sequences including SEMA4B, etc. can be used for search of compounds for diagnosis and treatment of lung cancer, based on the gene chip analysis (WO 02/86443). It is reported that NOV7 having 93% homology with SEMA4B on an amino acid level is overexpressed in cancer (WO 02/06329).

Please replace the paragraph bridging page 8, line 33, to page 9, line 2, with the following amended paragraph:

Homology of the amino acid sequences can be measured under the following conditions (an expectation value=10; gaps are allowed; matrix=BLOSUM62; filtering=OFF) using a homology scoring algorithm NCBI BLAST™ (National Center for Biotechnology Information Basic Local Alignment Search Tool).

Please replace the paragraph at page 23, lines 21-31, with the following amended paragraph:

When the protein of the present invention is extracted from the bacteria or cells, the bacteria or cell is collected after culturing by a publicly known method and suspended in an

appropriate buffer. The bacteria or cell is then disrupted by publicly known methods such as ultrasonication, a treatment with lysozyme and/or freeze-thaw cycling, followed by centrifugation, filtration, etc to produce crude extract of the protein. Thus, the crude extract of the protein can be obtained. The buffer used for the procedures may contain a protein modifier such as urea or guanidine hydrochloride, or a surfactant such as ~~Triton X-100~~TM TRITON X-100TM surfactant, etc. When the protein is secreted in the culture broth, the supernatant can be separated, after completion of the cultivation, from the bacteria or cell to collect the supernatant by a publicly known method.

Please replace the paragraph at page 70, lines 5-14, with the following amended paragraph:

The antisense oligonucleotide or the control oligonucleotide diluted in ~~Opti-MEM~~ OPTI-MEM® medium (Invitrogen Corp.) was mixed with ~~Oligofectamine~~ OLIGOFECTAMINETM reagent (Invitrogen Corp.) diluted with ~~Opti-MEM~~ OPTI-MEM® medium (Invitrogen Corp.) to 5-fold and settled at room temperature for 5 minutes, in a ratio of 8:3 (volume ratio). The resulting mixture was dispensed to the plate in 40 µL/well. The final concentration of the oligonucleotide was adjusted to become 250 nM. After incubation was continued for further 3 days under the conditions described above, the apoptosis induction activity of the two oligonucleotides above was assayed with Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics) in accordance with the protocol attached thereto.

Please replace the paragraph at page 71, lines 16-27, with the following amended paragraph:

Following the transfection, incubation was continued at 37° C for further 24 hours in a 5% carbon dioxide gas flow and the total RNA was then extracted by ~~RNeasy (registered trademark)~~ RNEASY® Mini Total RNA Kit (QIAGEN). Using as a template about 300 ng of the total RNA, reverse transcription was carried out on ~~TaqMan~~ TAQMAN® Reverse Transcription Reagents (Applied Biosystems) in accordance with the protocol attached thereto. Using as a template cDNA in an amount corresponding to 7 to 9 ng when converted into the total RNA, the number of expressed copies of SEMA4B, SEMA4B-M1, SEMA4B-M2 and SEMA4B-M3 genes was determined using two primers (SEQ ID NO: 17) and (SEQ ID NO: 18) and SYBR® Green PCR Master Mix (Applied Biosystems). The expression level of a gene for β -actin contained in the same amount of template cDNA was assayed on ~~TaqMan~~ TAQMAN® β -actin Control Reagents (Applied Biosystems), which was used as internal standard.

Please replace the paragraph at page 72, lines 11-28, with the following amended paragraph:

Using human lung cancer cell line (A549)-derived Marathon-Ready cDNA (CLONTECH) as a template, PCR was carried out by using two primers (SEQ ID NO: 19 and SEQ ID NO: 20). The reaction solution (50 μ l) was composed of 1 μ l of the above cDNA, 2.5 U of PfuTurbo-PFU TURBO® Hotstart DNA Polymerase (STRATAGENE), 1.0 μ M each of the primers (SEQ ID NO: 19 and SEQ ID NO: 20), 200 μ M of dNTPs and 25 μ l of 2x GC Buffer I (Takara Shuzo Co., Ltd.). PCR was carried out by reacting at 95° C for 1 minute and then repeating 30 times the cycle set to include 95° C for 1 minute, 60° C for 1 minute and 72° C for 4

minutes, and extension was performed at 72° C for 5 minutes. In order to add dATP to the PRC product at the 3' end, 5 U of ~~Ex-Taq~~ EX-TAQ® DNA Polymerase (Takara Shuzo Co., Ltd.) was added and the mixture was kept at 72° C. for 7 minutes. The PCR product obtained was purified using PCR Purification Kit (QIAGEN). The purified product was subcloned to plasmid vector pCR4-TOPO (Invitrogen Corp.) according to the protocol of TOPO TA PCR Cloning Kit (Invitrogen Corp.). The clones were transfected to Escherichia coli TOP10 and the clones bearing cDNA were selected in ampicillin-containing LB agar medium. The base sequences of individual clones were analyzed to give the base sequences of cDNAs represented by SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8 and SEQ ID NO: 11, respectively.

Please replace the paragraph at page 72, lines 29-34, with the following amended paragraph:

The base sequences in which the 1-237 base sequence and the 2749-3766 base sequence in the base sequence for SEMA4B gene (~~GenBank~~-GENBANKTM Accession No. XM_044533 gene) are added to the base sequences represented by SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8 and SEQ ID NO: 11 at the 5' and 3' ends thereof are shown by SEQ ID NO: 3, SEQ ID NO: 6, SEQ ID NO: 9 and SEQ ID NO: 12, respectively.

Please replace the paragraph bridging page 72, line 35, to page 73, line 2, with the following amended paragraph:

The amino acid sequence (SEQ ID NO: 1) encoded by the base sequence represented by SEQ ID NO: 2 completely coincided with SEMA4B protein encoded by SEMA4B gene (~~GenBank~~-GENBANKTM Accession No. XM_044533 gene).

Please replace the paragraph bridging page 74, line 33, to page 75, line 3, with the following amended paragraph:

Total RNA was prepared from the 91 cell lines described above using RNeasy RNEASY® Mini Total RNA Kit (QIAGEN). Reverse transcription was performed on the total RNA as a template using a random primer to prepare cDNA. Using this cDNA as a template, quantitative PCR was carried out to examine the expression levels of SEMA4B gene (SEQ ID NO: 2), SEMA4B-M1 gene (SEQ ID NO: 5), SEMA4B-M2 gene (SEQ ID NO: 8) and SEMA4B-M3 gene (SEQ ID NO: 11).

Please replace the paragraph at page 75, lines 4-10, with the following amended paragraph:

In the PCR above, the reaction was carried out under the same conditions as in EXAMPLE 3, using cDNA obtained from 3 to 4 ng of the total RNA described above as the template, and the copies of SEMA4B, SEMA4B-M1, SEMA4B-M2 and SEMA4B-M3 genes expressed were calculated. In parallel, the copy number of the gene for β -actin contained in 1 ng of the total RNA above was calculated using TaqMan™ TAQMAN™ Human β -actin Control Reagents (Applied Biosystems) and used as an internal standard.

Please replace the paragraph at page 77, lines 3-18, with the following amended paragraph:

SEMA4B gene was amplified by PCR using the plasmid SEMA4B/pCR4-TOPO obtained in EXAMPLE 4 as a template. In the reaction solution for the reaction, 2 ng of SEMA4B/pCR4-TOPO was used as a template and 2.5 U of Pfu Turbo PFU TURBO® Hotstart

DNA Polymerase (STRATAGENE), 1 μ M each of 2 primers (SEQ ID NO: 19 and SEQ ID NO: 21), 200 μ M of dNTPs and 5 μ l of 10x Pfu Buffer were added to make the solution volume 50 μ l. PCR was carried out by reacting at 95° C for 1 minute and then repeating 25 times the cycle set to include 95° C for 1 minute, 60° C for 1 minute and 72° C for 4 minutes. Next, the PCR product was purified using PCR Purification Kit (QIAGEN) and then treated with restriction enzymes XbaI and Eco RI. The plasmid p3xFLAG-CMV-14 (Sigma) was also treated with XbaI and Eco RI. Each DNA fragment was purified on PCR Purification Kit, followed by ligation using DNA Ligation Kit ver.2 (Takara Bio, Inc.). After the ligation solution was transfected to Escherichia coli TOP10, the transformed Escherichia coli was selected in ampicillin-containing LB agar medium. As a result of the analysis of individual clones, the plasmid pCMV-14-SEMA4B bearing the cDNA fragment corresponding to SEMA4B gene (SEQ ID NO: 2) was obtained.

Please replace the paragraph bridging page 78, line 23, to page 79, line 8, with the following amended paragraph:

One male rabbit KBL: JW (11 weeks old, Oriental Yeast Co., Ltd.) was used as an immunized animal. A suspension of complete Freund's adjuvant (Difco Laboratories) was used for primary sensitization and a suspension of incomplete adjuvant (Difco Laboratories) for the second sensitization and thereafter. The sensitization was performed by subcutaneous injection at the back and 0.5 mg of each antigen was used per sensitization. After the primary sensitization, it was repeated 3 times every 14 days. On day 52 after the primary sensitization, blood was collected through the carotid artery under anesthesia to give about 50 ml of serum. The serum thus obtained was concentrated by means of ammonium sulfate salting out. The total

amount of the crude IgG fractions obtained were purified on protein A-affinity column (Amersham-Bioscience Corp.) to give about 103 mg, about 76 mg, about 112 mg and about 122 mg of purified IgGs from Peptides 1, 2, 3 and 4, respectively. Furthermore, the IgG fractions bound to a column immobilized with the respective immunogen peptides were acquired. For the immobilization, the C-terminal Cys of each peptide was utilized and the peptide was coupled to ~~Sepharese~~ SEPHAROSE™ column (Amersham-Bioscience Corp.) using borate buffer. For elution from the column, 8M urea/phosphate buffered saline (PBS) was used. The eluate was dialyzed to PBS to remove urea, which was followed by ultraconcentration and sterilization by filtering. Thus, affinity-purified antibodies AS-2531, AS-2532, AS-2591 and AS-2592 to Peptides 1, 2, 3 and 4 were acquired in about 15 mg, about 126 mg, about 17 mg and about 35 mg, respectively.

Please replace the paragraph bridging page 79, line 12, to page 80, line 9, with the following amended paragraph:

SEMA4B protein (SEQ ID NO: 1) was detected using the purified peptide antibodies prepared in EXAMPLE 8. Human non-small lung cancer-derived NCI-H358 cells were suspended in 10 ml of RPMI-1640 medium (Invitrogen Corp.) containing 10% fetal calf serum (JRH) at a concentration of 1.5×10^6 and plated on a Petri dish of 10 cm in diameter. After incubation at 37° C overnight in a 5% carbon dioxide flow, 6 µg of the plasmid pCMV-14-SEMA4B prepared in EXAMPLE 6 was mixed with ~~Plus~~ PLUS™ reagent (Invitrogen Corp.) and OPTI-MEM I® medium (Invitrogen Corp.). After the mixture was allowed to stand at room temperature for 15 minutes, ~~LipofectAMINE~~ LIPOFECTAMINE™ Transfection Reagent (Invitrogen Corp.) and OPTI-MEM I® medium were added to the mixture, which was allowed to

stand at room temperature for further 15 minutes. The resulting mixture was dropwise added to the medium and incubation was continued. Two days after the transfection of expression plasmid, the cells were washed with ice-cooled PBS and 1 ml of ice-cooled RIPA buffer [50 mM Tris-hydrochloride buffer, pH 7.5, 150 mM sodium chloride, 1% ~~Triton X-100~~ TRITON X-100TM surfactant, 0.1% SDS, 1% deoxycholic acid, ~~CompleteTM~~ COMPLETETM tablet (Roche Diagnostics), Phosphatase Inhibitor Cocktail-2 (Sigma)] was added to the cells. The mixture was allowed to stand at 4° C for 30 minutes. This RIPA buffer was recovered and centrifuged at 15,000 rpm for 20 minutes. The supernatant obtained was used as the cell-free extract. This cell-free extract was mixed with a 2-fold concentration of SDS-PAGE sample buffer [125 mM Tris-hydrochloride buffer, pH 6.8, 40% glycerol, 4% SDS, 0.04% Bromophenol Blue and 5% 2-mercaptoethanol] in equal volumes. After heating at 95° C for 5 minutes, 10 µl of the mixture was provided for SDS-PAGE on 10% acrylamide gel. The protein separated by electrophoresis was transferred onto Clear Blotting P Membrane (ATTO) in a conventional manner, which was then allowed to stand in a blocking buffer [50 mM Tris-hydrochloride buffer, pH 7.5, 500 mM sodium chloride, 0.1% ~~Tween~~ TWEEN® 20, 5% skimmed milk] at room temperature for an hour. Next, the peptide antibody AS-2531, AS-2532, AS-2591 or AS-2592 produced in EXAMPLE 8 were diluted with the blocking buffer in a concentration of 3 µg/ml, followed by reacting at 4° C overnight. Subsequently, the reaction mixture was allowed to stand for an hour in a dilution of HRP-labeled anti-rabbit IgG antibody (Amersham-Bioscience Corp.) diluted in the blocking buffer to 50,000-fold or 100,000-fold. Detection was performed according to the protocol attached to ECL plus (Amersham-Bioscience Corp.). Thus, the SEMA4B protein was detected.

Please replace the paragraph at page 80, lines 19-35, with the following amended paragraph:

Using the plasmid pCMV-14-SEMA4B-3xFLAG acquired in EXAMPLE 7, the cell-free extract was prepared by the same procedures as in EXAMPLE 9. The cell-free extract, 400 μ l, was added to 50 μ l of a suspension of Protein G-Sepharose 4FF (Amersham-Bioscience Corp.) prepared by suspending in an equal volume of RIPA buffer) and 5 μ g of any one of the peptide antibodies AS-2531, AS-2532, AS-2591 and AS-2592 described in EXAMPLE 8 was further added thereto. The resulting mixture was agitated at 4° C overnight. After the Protein G-Sepharose 4FF co-precipitated fraction was washed with RIPA buffer, the fraction was suspended in 50 μ l of SDS-PAGE sample buffer [62.5 mM Tris-hydrochloride buffer, pH 6.8, 20% glycerol, 2% SDS, 0.02% Bromophenol Blue and 2.5% 2-mercaptoethanol]. After heating at 95° C for 5 minutes, 5 μ l or 10 μ l of the suspension was provided for SDS-PAGE on 10% acrylamide gel. Detection was performed by the same procedures as in EXAMPLE 9, except that mouse anti-FLAG-ANTI-FLAG® M2 antibody (Sigma) diluted with the blocking buffer to 0.2 μ g/ml or 0.1 μ g/ml was used as a primary antibody and HRP-labeled anti-mouse IgG antibody (Amersham-Bioscience Corp.) diluted with the blocking buffer to 25,000-fold or 50,000-fold was used as a secondary antibody.

Please replace the paragraph at page 81, lines 9-33, with the following amended paragraph:

Lung cancer cell lines NCI-H2228, NCI-H1651, NCI-H358, NCI-H23 and NCI-H1703; ovary cancer cell lines SKOV-3 and TOV-21G; prostate cancer cell line DU145; and pancreatic cancer cell line PANC-1 were plated, respectively, on two Petri dishes of 10 cm in diameter. For

each of the cells, the cells for one Petri dish were dispersed in Trypsin-EDTA and the number of cells was counted. Based on the cells counted, ice-cooled RIPA buffer (described in EXAMPLE 9) was added to the remaining one Petri dish in $1 \text{ ml}/5 \times 10^6$ cells, followed by allowing to stand at 4°C for 30 minutes. This RIPA buffer was recovered and centrifuged at 15,000 rpm for 20 minutes. The supernatant obtained was used as the cell-free extract. Meanwhile, a resin obtained by crosslinking the peptide antibody AS-2531 described in EXAMPLE 8 with Protein G-Sepharose 4FF (Amersham-Bioscience Corp.) according to the protocol attached to SizeTM X Protein G Immunoprecipitation Kit (Pierce Chemical) was prepared and suspended in an equal volume of RIPA buffer. The aforesaid cell-free extract, 400 μl , was added to 30 μl of this suspension, followed by agitation overnight at 4°C . After washing the Protein G-Sepharose 4FF co-precipitated fraction with RIPA buffer, the fraction was suspended in 30 μl of SDS-PAGE sample buffer described in EXAMPLE 10 and the suspension was heated at 95°C for 5 minutes. Then, 20 μl of the suspension was provided for SDS-PAGE on 10% acrylamide gel. Using the peptide antibody AS-2532, detection was performed in a manner similar to EXAMPLE 9.

Please replace the paragraph at page 82, lines 2 to 34, with the following amended paragraph:

Human non-small cell lung cancer-derived NCI-H358 was suspended in 2 ml of RPMI-1640 medium (Invitrogen Corp.) containing 10% fetal calf serum (JRH), 1 mM sodium pyruvate and 25 mM HEPES. The suspension was plated on a 6-well plate, followed by incubation overnight at 37°C in a 5% carbon dioxide gas. On the other hand, 1 μg of plasmid pCMV-14-SEMA4B described in EXAMPLE 6, which was diluted with the OPTI-MEM 1® medium

(Invitrogen Corp.), was mixed with 6 μ l of ~~Plus~~ PLUSTM reagent (Invitrogen Corp.) and the mixture was allowed to stand at room temperature for 15 minutes. Then, 4 μ l of ~~LipofectAMINE~~ LIPOFECTAMINETM reagent (Invitrogen Corp.) diluted in OPTI-MEM I® medium was added to the mixture, which was allowed to stand at room temperature for further 15 minutes. The mixture was dropwise added to the medium and incubation was further continued for a day. The cells were then dispersed in trypsin-EDTA (Invitrogen Corp.) and diluted to 10-fold in the above medium added with G418 (Promega Corp.) in 400 μ g/ml, followed by plating which was plated on a 24-well plate. While the medium was exchanged with the G418-containing medium (G418 selection medium) every 3 or 4 other days, incubation was continued at 37° C in a 5% carbon dioxide gas flow. From colonies formed when one to three cells proliferated, the cells were recovered and plated equally on two wells of a 48-well plate. After incubation was continued until the cell density reached 50% or more, 50 μ l of the SDS-PAGE sample buffer described in EXAMPLE 10 was added to the cells for one well to prepare the cell lysate. After heat treatment at 95° C for 5 minutes, 5 μ l was provided on for SDS-PAGE on 10% acrylamide gel. Using the peptide antibody AS-2532, western blotting was performed by a modification of the procedures described in EXAMPLE 9 to explore a stable cell line constitutively expressing the SEMA4B-A protein (SEQ ID NO: 1). The cells recovered from the other well were diluted in 0.7 cell/well and then plated on a 96-well plate. While exchanging the G418 selection medium every 3 or 4 other days, incubation was continued at 37° C in a 5% carbon dioxide gas flow until the cell density reached about 50%. Again, the cells were plated equally on 2 wells of a 48-well plate, and incubation was continued until the cell density reached 50% or more. Using the cell lysate prepared from the cells for one well, western blotting was performed as described above. A clone with the highest expression of SEMA4B

protein (SEQ ID NO: 1) was selected to acquire SEMA4B/H358 as the cell line stably expressing SEMA4B.

Please replace the paragraph at page 84, lines 7-22, with the following amended paragraph:

NCI-H358 was suspended in RPMI-1640 medium (Invitrogen Corp.) containing 10% fetal calf serum (JRH), 1 mM sodium pyruvate and 25 mM HEPES. NCI-H358 was plated on a 96-well flat bottomed tissue culture plate (BD Falcon) at a cell density of 8×10^3 /well (80 μ l of medium volume), followed by incubation at 37° C overnight in a 5% carbon dioxide gas flow. On the other hand, 0.06 μ g each of the oligonucleotide (SEQ ID NO: 13) described in EXAMPLE 2 and control oligonucleotide (SEQ ID NO: 14) were diluted in OPTI-MEM I® medium (Invitrogen Corp.). The dilution was mixed with 0.5 μ l of ~~Plus~~ PLUSTM reagent (Invitrogen Corp.) and the mixture was allowed to stand at room temperature for 15 minutes. To the mixture, 0.4 μ l of ~~LipofectAMINE~~ LIPOFECTAMINETM transfection reagent (Invitrogen Corp.) diluted in OPTI-MEM I® medium was added. The mixture was allowed to stand at room temperature for further 15 minutes. The whole volume of the mixture was added to the medium for NCI-H358, and incubation was continued for further 3 hours. Following the protocols attached to Cell Death Detection ELISA^{PLUS} (Roche Diagnostics) and ~~Caspase-Glo~~ CASPASE-GLO® 3/7 assay (Promega Corp.), the oligonucleotide described above was assayed for its apoptosis induction activity.

Please replace the paragraph at page 84, lines 23-27, with the following amended paragraph:

As a result, the oligonucleotide showed the apoptosis induction activity in NCI-H358 as higher by 1.42 times and 1.77 times, respectively, than the control antisense oligonucleotide used as a negative control by both Cell Death Detection ELISA^{PLUS} and ~~Caspase-Glo~~ CASPASE-GLO® 3/7 assay, indicating that there was a statistically significant difference ($P \leq 0.01$) (Tables 5 and 6).

Please replace the paragraph bridging page 85, line 11, to page 86, line 9, with the following amended paragraph:

For NCI-H2228, RPMI-1640 medium (Invitrogen Corp.) containing 10% fetal calf serum (JRH), 1 mM sodium pyruvate and 25 mM HEPES was used. For NCI-H1651, ACL-4 medium (ATCC) containing 10% FBS was used. For NCI-H23, RPMI-1640 medium (Invitrogen Corp.) containing 10% fetal calf serum (JRH) and 25 mM HEPES was used. The respective cells were suspended in the corresponding media and plated on a 96-well flat bottomed tissue culture plate (BD Falcon) at cell densities of 7.5×10^3 /well (NCI-H2228), 7.5×10^3 /well (NCI-H1651) and 5×10^3 /well (NCI-H23), respectively (125 μ l of medium volume), followed by incubation overnight at 37° C in a 5% carbon dioxide gas flow. On the other hand, 0.135 μ g each of the antisense oligonucleotide (SEQ ID NO: 13) described in EXAMPLE 2 and the control oligonucleotide (SEQ ID NO: 14) were diluted in OPTI-MEM I® medium (Invitrogen Corp.), respectively. After each dilution was mixed with 0.75 μ l of ~~Plus~~ PLUS™ reagent (Invitrogen Corp.), the mixture was allowed to stand at room temperature for 15 minutes. Then, 0.4 μ l of ~~LipofectAMINE~~ LIPOFECTAMINE™ reagent (Invitrogen Corp.) diluted in OPTI-MEM I®

medium was added to the mixture, which was allowed to stand at room temperature for further 15 minutes. The whole volume of the mixture was added to the medium and incubation was further continued for 3 days. Following the protocol attached to Cell Death Detection ELISA^{PLUS} (Roche Diagnostics), the oligonucleotide described above was assayed for its apoptosis induction activity.